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Mechanism of Action of Cephalosporins and Resistance Caused by Decreased Affinity for Penicillin-Binding Proteins in *Bacteroides fragilis*

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The susceptibilities of 52 clinical isolates of *Bacteroides fragilis* to five monoanionic cephalosporins were examined. Cefoperazone showed the highest antibacterial activity, followed by ceftezole, cefazolin, cefamandole, and cephalothin. There were two groups of resistant strains: one group (ca. 15%), of which *B. fragilis* G-232 was a typical sample, was resistant to ceftezole (MIC, 100 μg/ml), cefazolin (MIC, 100 μg/ml), and cephalothin (MIC, 200 μg/ml) but not cefoperazone (MIC, 6.25 μg/ml) or cefamandole (MIC, 25 μg/ml). On the basis of studies of stability to β-lactamase, outer membrane permeation, and affinity for penicillin-binding proteins (PBPs), we conclude that decreased affinity for PBP 3 may play an important role in the resistance to ceftezole, cefazolin, and cephalothin in *B. fragilis* G-232. Another group (also ca. 15%), of which *B. fragilis* G-242 was a representative, was resistant to all five cephalosporins (MIC, 100 to 400 μg/ml) and produced a high amount of β-lactamase. Similar broad-spectrum resistance was seen in a mutant of strain G-232 that had a greater-than-30-fold increase in β-lactamase production.

β-Lactam antibiotics owe their antibacterial activity to a reaction with one or more penicillin-binding proteins (PBPs) which act as transpeptidases in completing the peptidoglycan network of the cell wall (2, 26). The PBPs are believed to be attached to the outer surface of the cytoplasmic membrane. Gram-negative bacteria, however, are covered by an outer membrane which acts as a permeation barrier to various toxic materials, such as β-lactam antibiotics and detergents (14). β-Lactam antibiotics must penetrate this outer membrane before they can reach their target PBPs. Moreover, a β-lactamase which can hydrolyze β-lactam antibiotics before they reach their target PBPs exists in the periplasm space between the inner and outer membranes (12). Therefore, β-lactam antibiotics must be resistant to β-lactamase hydrolysis.

Bacteroides fragilis is an anaerobe frequently recovered from human infections, especially intra-abdominal infections (5, 15). *B. fragilis* produces β-lactamase, which may play an important role in its resistance to β-lactam antibiotics (18, 22, 24, 27, 31). In this study, we used a series of monoanionic cephalosporins which have different hydrophilicities (cefoperazone, ceftezole, cefazolin, cefamandole, and cephalothin), because it was therefore possible to use them to assay outer membrane permeation. We also examined the affinities of these drugs for PBPs, their outer membrane permeations, and stabilities to β-lactamase in *B. fragilis*.

MATERIALS AND METHODS

Bacterial strains. Fifty-two strains of *B. fragilis* were isolated from clinical specimens at the School of Medicine, Showa University, Tokyo, Japan; Kawasaki City Hospital, Kawasaki, Japan; Tajimi City Hospital, Tajimi, Japan; School of Medicine, Gifu University, Gifu, Japan; and Kawasaki Medical School, Kurashiki, Japan, from 1979 to 1980. *B. fragilis* G-253 R and G-232 R, which produce a large amount of β-lactamase, were selected from G-253 and G-232 on the basis of ampicillin resistance after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Aldrich Chemical Co., Inc., Milwaukee, Wis.). NTG treatment was performed as follows. A 1-ml sample of overnight culture in Gifu Anaerobic Medium (GAM) broth was harvested by centrifugation, washed once in 0.05 M phosphate buffer (pH 7.0), and resuspended in the same buffer. NTG was added (final concentration, 100 μg/ml), and incubation was continued for 30 min at 37°C. The cells were harvested by centrifugation, washed three times, and resuspended in the same buffer. Then, 9 ml of GAM broth was added and incubation was continued overnight at 37°C. GAM agar containing four times the MIC of ampicillin was used as the selection medium. These strains were stored in skim milk (10%) at −70°C.

Drugs. The drugs used in this study were all commercially available. Their sources were as follows: cefamandole and cephaloridine, Shionogi Chemical Co., Ltd., Osaka, Japan; cefazolin and ceftezole, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan; cefoperazone, Toyama Chemical Co., Ltd., Tokyo, Japan; cephalothin, Torii Pharmaceutical Co., Ltd., Tokyo, Japan; chloramphenicol, Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan; norfloxacin, Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan; and tetracycline, Takeda Chemical Industries, Ltd., Osaka, Japan.

Media. GAM broth and GAM agar were used for culture. These media were produced by Nissui Pharmaceutical Co., Ltd., Tokyo, Japan.

Antibiotic susceptibility test. Antibiotic susceptibility was determined by an agar dilution method described previously (30). Plates containing serial twofold dilutions of antibiotic were inoculated with one loopful (about 5 μl) of approximately 2×10^6 CFU/ml of suitable diluted overnight cultures in GAM broth at 37°C. The MICs (micrograms per milliliter)

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were scored after 18 h of incubation at 37°C in an anaerobic system (model 1024; Forma Scientific, Marietta, Ohio).

Preparation of crude β-lactamase. For the preparation of crude β-lactamase, we followed the method described previously (30).

Preparation of outer membrane proteins. Cultures (100 ml) in the late-logarithmic phase of growth were harvested by centrifugation at 5,000 × *g* for 15 min at 4°C. Cells were washed once and recentrifuged. The cell suspension was sonicated in an ultrasonic disrupter. The disrupted cell suspension was centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant was ultracentrifuged at 100,000 × *g* for 30 min at 4°C, washed once, and then used as the outer membrane proteins. Outer membrane proteins were detected by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (8).

β-Lactamase assay. β-Lactamase activity was assayed by a modification of the microiodometric method of Novick (16) or by a direct spectrophotometric method (30). One unit of β-lactamase activity was equal to 1 micromole of substrate hydrolyzed per minute at 30°C in 0.05 M phosphate buffer (pH 7.0).

Protein assay. For the protein assay, we followed the method of Lowry et al. (10).

Reverse-phase TLC. The hydrophobic character of the cephalosporins was expressed as the R_f value, which was measured by reverse-phase thin-layer chromatography (TLC). The polar mobile phase was acetate-Veronal buffer (pH 7.0):methanol (4:1, vol/vol). Merck TLC silica gel 60 F_{254} siliconized precoated plates were used as the nonpolar stationary phase. A sample was dissolved in the acetate-Veronal buffer to give about 3 mg/ml, and 1 to 2 μl of the solution was loaded on the TLC plate.

Assay of outer membrane permeation by cephalosporins. The assay of outer membrane permeation by cephalosporins was done as described previously (23, 33), except that the bacterial cells were grown in GAM broth. Cultures (200 ml) in the mid-logarithmic phase of growth were harvested by centrifugation at 5,000 × *g* for 15 min at 20°C. Cells were washed once with 0.1 M phosphate buffer (pH 7.0) containing 1 mM magnesium sulfate and resuspended in 30 ml of the same buffer. A portion of the cell suspension was sonicated for 2 min at 4°C by using an ultrasonic disrupter. This suspension was used to measure the velocity of hydrolysis by disrupted cells ($V_{disrupt}$). The rest of the cell suspension was used for the direct measurement of the velocity of hydrolysis by the intact cell suspension (V_{intact}). At the same time that V_{intact} was measured, the intact cell suspension was centrifuged quickly for 2 min and β-lactamase activity of the suspension (V_{sup}) was measured.

Permeation parameter C (13, 29) was calculated as follows:

$$C = (1/S_0 - S_i)\,[V_{max}\,S_i/(K_m + S_i)]$$
$$S_i = (V_i/V_d)\,\{K_m\,S_0/[K_m + S_0 - S_0(V_i/V_d)]\}$$
$$V_{max} = (1 + K_m/S_0)V_d$$

where $V_d = V_{disrupt} - V_{sup}$ and $V_i = V_{intact} - V_{sup}$. V_{max} and K_m are the maximum velocities of hydrolysis of a test β-lactam antibiotic and the Michaelis constant, respectively. S_0 and S_i are concentrations of β-lactam antibiotics in the medium and in the periplasmic space of the cells, respectively, at steady state. The permeation parameter was normalized by the dry weight of cells in the assay mixture.

Assay of PBPs. For the assay of PBPs, we essentially followed the procedure described previously (25). Cultures (2 liters) in the late-logarithmic phase of growth were harvested by centrifugation at 5,000 × *g* for 15 min at 4°C. Cells were washed once, sonicated, and recentrifuged. The supernatant was ultracentrifuged at 100,000 × *g* for 30 min at 4°C, washed twice, and then used as envelope preparations. The envelope preparations were adjusted to a protein concentration of 20 mg/ml and were incubated with the appropriate β-lactam antibiotic at 30°C for 10 min in a total volume of 33 μl. Then 0.15 μCi of [^{14}C]benzylpenicillin (specific activity, 59 mCi/mol; Amersham International plc, Buckinghamshire, England) was added, and incubation was continued for 10 min. PBPs were detected by SDS–7.5% polyacrylamide gel electrophoresis (8) and fluorography (9).

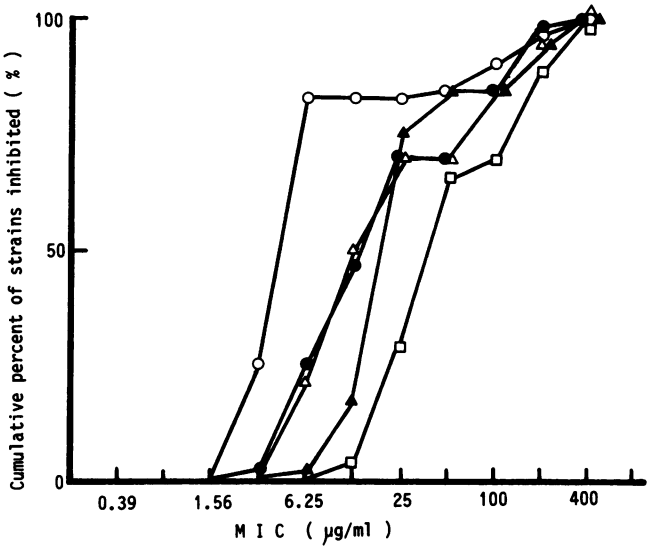

FIG. 1. Distribution of susceptibilities of 52 *B. fragilis* strains to cephalosporins. Symbols: ○, cefoperazone; ●, ceftezole; △, cefazolin; ▲, cefamandole; □, cephalothin.

Morphological response of bacteria. A 5% inoculum for an overnight culture was used to inoculate GAM broth, which was then incubated statically at 37°C for 2 h. Cultures were divided at about 10^8 cells per ml, and test antibiotics were added to give final concentrations equal to the MIC. After 2 h of incubation, samples were withdrawn and placed on a 1.5% agar-overlaid slide and examined with a phase-contrast microscope (model MTD; Nihon Kougaku Co., Ltd., Tokyo, Japan).

RESULTS

Antibacterial activity of cephalosporins. The susceptibilities of 52 *B. fragilis* strains to the five cephalosporins are shown in Fig. 1. Cefoperazone showed the most-potent antibacterial activity, followed by ceftezole, cefazolin, cefamandole, and cephalothin. A typical susceptible strain was *B. fragilis* G-253, with MICs and β-lactamase production as shown in Table 1.

There were two groups of resistant strains. One group (8 of 52 *B. fragilis* strains [ca. 15%]), including *B. fragilis* G-232, was resistant to ceftezole (MIC, 100 μg/ml), cefazolin (MIC, 100 μg/ml), and cephalothin (MIC, 200 μg/ml) but was not resistant to cefoperazone (MIC, 6.25 μg/ml) or cefamandole (MIC, 25 μg/ml). These strains produced a small amount of β-lactamase. Another group (8 of 52 *B. fragilis* strains [ca. 15%]), including *B. fragilis* G-242, was resistant to all five cephalosporins (MIC, 100 to 400 μg/ml). These strains produced large amounts of β-lactamase.

TABLE 1. Susceptibilities of *B. fragilis* strains to various antimicrobial agents

B. fragilis strain	MIC (μg/ml)[a]							β-Lactamase activity[b] (U/mg of protein)
	CPZ	CTZ	CEZ	CMD	CET	APC	NFLX	
G-253	3.13	12.5	12.5	12.5	25	6.25	50	0.02
G-253 R	6.25	25	25	25	50	12.5	50	0.08
G-232	6.25	100	100	25	200	12.5	25	0.02
G-232 R	200	400	400	400	400	200	25	0.62
G-242	100	200	400	400	400	100	25	1.05

[a] MICs were determined by an agar dilution method with one loopful of approximately 2×10^6 CFU/ml. Abbreviations: CPZ, cefoperazone; CTZ, ceftezole; CEZ, cefazolin; CMD, cefamandole; CET, cephalothin; APC, ampicillin; NFLX, norfloxacin. The MICs of chloramphenicol and tetracycline were 3.13 and 25 μg/ml, respectively, for all strains.

[b] β-Lactamase activities were assayed by using 100 μM cephaloridine as substrate.

Table 1 also shows the susceptibilities of *B. fragilis* G-253 R and G-232 R, which were used to assay outer membrane permeation. They showed decreased susceptibility to β-lactam antibiotics, probably because the amounts of β-lactamase produced by G-253 R and G-232 R were 4- and 31-fold greater than the amounts produced by G-253 and G-232, respectively. However, their susceptibilities to norfloxacin, chloramphenicol, and tetracycline were not changed, suggesting that outer membrane permeations of G-253 R and G-232 R were similar to those of G-253 and G-232.

The substrate profiles of β-lactamase from parental (G-253, G-232) and mutant (G-253 R, G-232 R) strains, together with those of G-242, were examined. All were typical species-specific *B. fragilis* cephalosporinases. There was no obvious difference in substrate profile between parental and mutant strains. We postulated that the reason why some strains, such as G-232, were resistant to ceftezole, cefazolin, and cephalothin was that these drugs might be hydrolyzed more easily than cefoperazone and cefamandole. However, this proved not to be the case.

Outer membrane permeation by cephalosporins. We selected *B. fragilis* G-253 R and G-232 R to assay outer membrane permeation because both strains produced cephalosporinase constitutively. There was no obvious difference in the outer membrane protein profiles between the parental (G-253, G-232) and mutant (G-253 R, G-232 R) strains (Fig. 2). The relationship between outer membrane permeation and hydrophilicity of the antibiotics is shown in Table 2.

Hydrophilicity of cefoperazone was the lowest, followed by cephalothin, cefamandole, cefazolin, and ceftezole. The permeations by the cephalosporins were nearly equal; i.e., permeation parameters C were 0.62×10^{-6} to 1.35×10^{-6} cm^3/min per μg of dry cell weight in *B. fragilis* G-253 R and 6.6×10^{-6} to 11.0×10^{-6} cm^3/min per μg of dry cell weight in *B. fragilis* G-232 R. There was no correlation between the MICs of the cephalosporins and outer membrane permeation.

Affinity of cephalosporins for PBPs. Examination by SDS-polyacrylamide gel electrophoresis showed that *B. fragilis* G-253 and G-232 had five high-molecular-weight (MW) PBPs: PBP 1a (MW, 94,000), PBP 1b (MW, 90,000), PBP 1c (MW, 88,000), PBP 2 (MW, 82,000), and PBP3 (MW, 72,000) (Fig. 3).

The affinities of cephalosporins for the PBPs from *B. fragilis* G-253 and G-232 were examined by using envelope preparations. The gels from competition assays were examined visually and by densitometer, and the I_{50} values (concentrations at which the density of the autoradiograph band was decreased by 50%) were adjusted to equate with the nearest value used in an MIC scheme (e.g, 0.34 would become 0.39 μg/ml, 1.70 would become 1.56 μg/ml).

As shown in Table 3, although the five cephalosporins had no affinity for PBP 1a of *B. fragilis* G-253, they had good affinity for PBPs 1b, 1c, 2, and 3. Cefoperazone bound most strongly to PBPs 1b and 1c at 0.78 μg/ml, and the other four cephalosporins bound to those PBPs at 6.25 to 12.5 μg/ml. The binding affinity of cefoperazone for PBP 2 was the strongest (0.39 μg/ml), followed by cefamandole (0.78 μg/ml), whereas those of cephalothin, ceftezole, and cefazolin were weak (6.25 to 50 μg/ml). At a concentration approximating the MIC, cefoperazone caused the most filamentation, followed by cefamandole, whereas no filamentation was seen with cephalothin, ceftezole, or cefazolin (Fig. 4). A correlation was thus observed between binding affinity of cephalosporins for PBP 2 and filamentation. All five cephalosporins bound strongly to PBP 3 at 0.1 to 0.2 μg/ml.

Table 4 shows the competition of cephalosporins for the

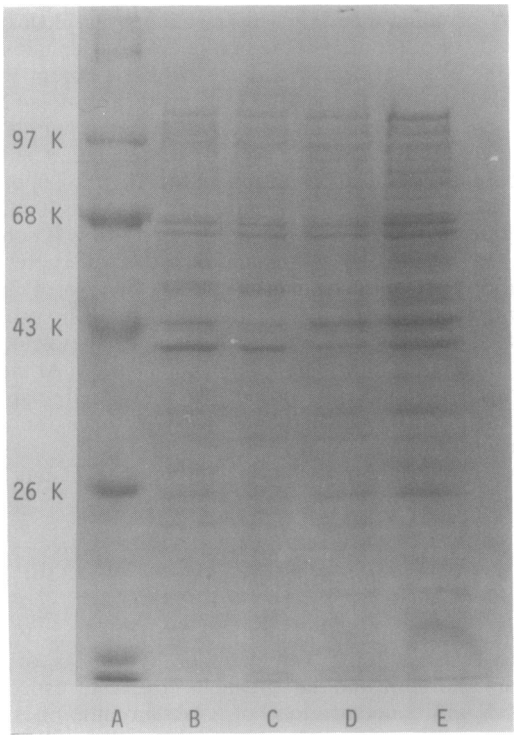

FIG. 2. SDS-polyacrylamide gel electrophoresis of outer membrane proteins. Proteins were prepared from cell envelopes as described in the text and analyzed on an SDS–10% polyacrylamide slab gel. Lanes: A, standard proteins; B, *B. fragilis* G-253; C, *B. fragilis* G-253 R; D, *B. fragilis* G-232; E, *B. fragilis* G-232 R. Molecular weights (10^3) are shown on the left.

TABLE 2. Outer membrane permeability of *B. fragilis* by cephalosporins

Antibiotic	Hydrophilicity[a]	Permeability parameter (10^{-6} cm^3/min per μg of dry cell wt)	
		G-253 R	G-232 R
Cefoperazone	0.35	0.62	6.7
Cephalothin	0.40	1.12	11.0
Cefamandole	0.48	1.35	7.0
Cefazolin	0.71	0.84	6.6
Ceftezole	0.80	0.78	10.0

[a] The hydrophilicity is expressed as the R_f value as measured by reverse-phase TLC.

PBPs of *B. fragilis* G-232. As with strain G-232, none of the cephalosporins had affinity for PBP 1a of *B. fragilis* G-232. For PBPs 1b and 1c, cefoperazone bound most strongly at 0.78 and 0.39 μg/ml, and the other four cephalosporins bound at 3.13 to 12.5 μg/ml. The binding affinities of cefamandole and cefoperazone for PBP 2 were strong (0.2 and 0.39 μg/ml), followed by cephalothin, whereas ceftezole and cefazolin had no affinity for PBP 2. All five cephalosporins bound strongly to PBP 3 at 0.2 to 1.56 μg/ml. Cefoperazone and cefamandole bound strongly to PBP 3 of *B. fragilis* G-232 and G-253 at 0.2 to 0.39 μg/ml. Ceftezole, cefazolin, and cephalothin bound to PBP 3 of G-232 at 1.56 μg/ml. The binding affinities of these cephalosporins for G-232 were 8- to 16-fold lower than those for G-253. A correlation was observed between the magnitude of the decrease in MICs and the magnitude of the decrease in affinity for PBP 3.

DISCUSSION

On the basis of studies of their stabilities to β-lactamase, outer membrane permeations, and affinities for PBPs, we conclude that the affinity of cephalosporins for high-molecular-weight PBPs may play the most important role in the antibacterial activity of cephalosporins for susceptible strain *B. fragilis* G-253. PBPs from *B. fragilis* have been studied by

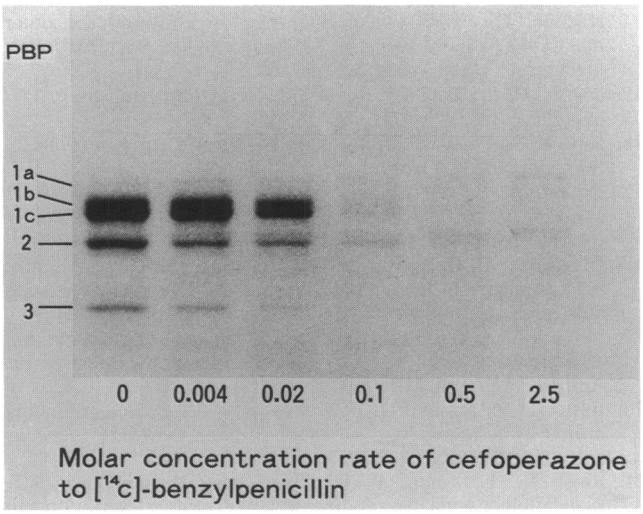

FIG. 3. Competitive inhibition of cefoperazone for [^{14}C]benzylpenicillin binding to *B. fragilis* G-253 PBPs. The ratios of cefoperazone concentrations to [^{14}C]benzylpenicillin concentrations are shown on the abscissa.

TABLE 3. Competition of cephalosporins for PBPs of *B. fragilis* G-253 envelope preparations

Antibiotic	I_{50}[a] (μg/ml) for PBP:			
	1b	1c	2	3
Cefoperazone	0.78	0.78	0.39	0.20
Ceftezole	12.5	6.25	50	0.10
Cefazolin	12.5	6.25	12.5	0.10
Cefamandole	6.25	6.25	0.78	0.20
Cephalothin	6.25	6.25	6.25	0.20

[a] I_{50}, Concentration at which the density of the autoradiograph band was decreased by 50%. The I_{50}s of all antibiotics for PBP 1a were >100 μg/ml.

several groups (6, 19), and similar profiles of these high-molecular-weight PBPs have been obtained. Piddock and Wise demonstrated that the high-molecular-weight PBPs were involved in binding at concentrations approximating the MICs of β-lactam antibiotics (19). Georgopapadakou et al. demonstrated that PBP 2 had the highest affinity for β-lactam antibiotics and that binding correlated with MICs (6).

On the other hand, many mechanisms by which bacterial strains become resistant to β-lactam antibiotics have been postulated. *B. fragilis* G-232 was resistant to ceftezole, cefazolin, and cephalothin, but it was not resistant to cefoperazone or cefamandole. We studied the mechanisms of resistance to ceftezole, cefazolin, and cephalothin in *B. fragilis* G-232.

First, *B. fragilis* G-232 produced a small amount of β-lactamase, and there did not seem to be any differences in the stabilities of the different cephalosporins to this β-lactamase. Therefore, β-lactamase hydrolysis did not contribute to resistance to ceftezole, cefazolin, and cephalothin in *B. fragilis* G-232. Second, the outer membrane permeation by cephalosporins was higher in *B. fragilis* G-232 than in G-253. No correlation was observed between MICs and permeation by cephalosporins. Therefore permeation did not contribute to ceftezole, cefazolin, and cephalothin resistance in *B. fragilis* G-232. Third, all five cephalosporins had good affinity for the high-molecular-weight PBPs in *B. fragilis* G-232, as well as G-253, and a correlation was observed between the magnitude of the decrease in MICs and the magnitude of the decrease in affinity for PBP 3. On the basis of these studies of stability to β-lactamase, outer membrane permeation, and affinity for PBPs, we conclude that decreased affinity for PBP 3 may play an important role in the mechanisms of resistance to ceftezole, cefazolin, and cephalothin in *B. fragilis* G-232.

Furthermore, *B. fragilis* G-242 was resistant to all five cephalosporins. *B. fragilis* G-242 produced a large amount of β-lactamase. In a previous paper, we reported that the outer

TABLE 4. Competition of cephalosporins for PBPs of *B. fragilis* G-232 envelope preparations

Antibiotic	I_{50}[a] (μg/ml) for PBP:			
	1b	1c	2	3
Cefoperazone	0.78	0.39	0.39	0.20
Ceftezole	12.5	6.25	>100	1.56
Cefazolin	12.5	6.25	>100	1.56
Cefamandole	6.25	3.13	0.20	0.39
Cephalothin	6.25	3.13	1.56	1.56

[a] I_{50}, Concentration at which the density of the autoradiograph band was decreased by 50%. The I_{50}s of all antibiotics for PBP 1a were >100 μg/ml.

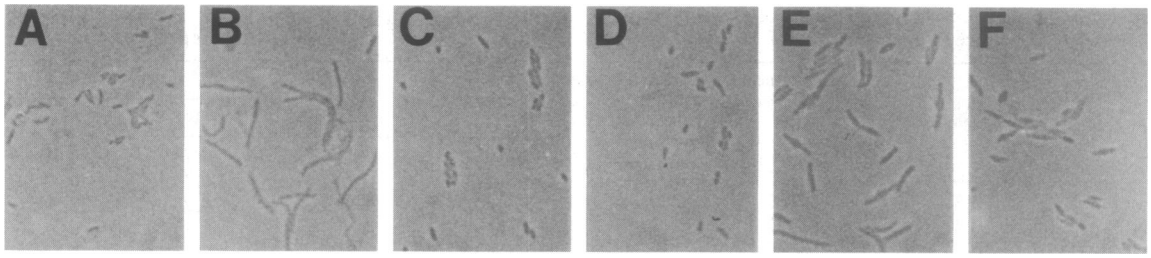

FIG. 4. Morphological response of *B. fragilis* G-253 by phase-contrast microscopy. A, Control; B, cefoperazone; C, ceftezole; D, cefazolin; E, cefamandole; F, cephalothin.

membrane permeations by the five cephalosporins in *B. fragilis* G-242 were nearly equal (32). There are no data concerning the affinity for PBPs in *B. fragilis* G-242 because [^{14}C]benzylpenicillin was hydrolyzed by β-lactamase. We conclude that β-lactamase hydrolysis plays the greatest role in cephalosporin resistance by *B. fragilis* G-242. Similar broad-spectrum resistance was seen in a mutant of strain G-232 that had a greater-than-30-fold increase in β-lactamase production.

It is known that changes in the affinity or quantity of PBPs play a major role in the development of β-lactam resistance among clinical isolates of bacteria, such as methicillin-resistant *Staphylococcus aureus* (20, 21), non-β-lactamase-producing penicillin-resistant *Neisseria gonorrhoeae* (1, 4), and *Pseudomonas aeruginosa* (7, 11). Eight of 52 *B. fragilis* strains (ca. 15%), including G-232, were resistant because of decreased affinity for PBPs. It is also well known that β-lactamase may play an important role in the mechanisms of resistance to β-lactam antibiotics among clinical isolates of bacteria, including *B. fragilis* (12, 18, 22, 24, 27, 31). Resistance in 8 of 52 *B. fragilis* strains (ca. 15%), such as G-242, was the result of β-lactamase hydrolysis. Furthermore, resistance also is derived from changes in outer membrane permeation among gram-negative bacteria such as *Enterobacter cloacae* (3, 28). Olsson et al. showed that limited outer membrane permeation in *B. fragilis* may contribute to β-lactam antibiotic resistance (17), but in our observation no strains of *B. fragilis* were resistant because of decreased permeation.

The strains of *B. fragilis* used in this study were fully pathogenic and were recovered from human infections. The increased use of β-lactam antibiotics will result in new strategies by organisms to overcome the drug challenge. Resistance to β-lactam antibiotics in *B. fragilis* probably will continue to be a problem in the foreseeable future.

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